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Periadolescent exposure to ethanol and diazepam alters the aversive properties of ethanol in adult mice

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Abstract

Evidence suggests that the developing adolescent brain may be especially vulnerable to long-term neurobehavioral consequences following ethanol exposure and withdrawal. In the present study, we examined the long-term effect of adolescent ethanol withdrawal on a subsequent EtOH-induced conditioned taste aversion (CTA). Periadolescent and adult C3H mice were exposed to 64 h of continuous (single withdrawal) or intermittent (multiple withdrawal) ethanol vapor. Following each ethanol exposure, animals received either 0, 1, 2, or 3 mg/kg diazepam (DZP) in an attempt to counteract the possible effect of ethanol withdrawal. About 6 weeks following ethanol and DZP treatment, animals were tested for an EtOH-induced CTA. As expected, exposure to EtOH during adolescence attenuated the EtOH-induced CTA as compared to controls. Unexpectedly, administration of DZP during withdrawal did not spare but rather mimicked the attenuation of the EtOH-induced CTA seen in animals exposed to ethanol in adolescence. This attenuation was not evident when EtOH and/or DZP was administered in adulthood. Given the similar mode of action of EtOH and DZP on the GABA system, the principal implication of the present findings is that the intoxicating effect of ethanol on the developing brain can result in long-term changes in the aversive properties of EtOH.
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Keywords: EtOH; Adolescent exposure; Withdrawal; Diazepam; Conditioned taste aversion; Mice

1. Introduction

Alcohol consumption and intoxication are common among the adolescent population. The National Adolescent Survey, reports that 25.9% of eighth-graders and 47.2% of tenth-graders consumed an intoxicating level of alcohol at least once in their lifetime (Windle, 1990). More recent findings from the Monitoring the Future survey indicate that 30% of individuals in the 12th grade reported consuming five or more drinks, consecutively, in the most recent 2 weeks before the survey (Johnston et al., 2005). Perhaps even more startling are epidemiological studies suggesting that the age at which alcohol experimentation is initiated may profoundly impact the likelihood of developing substance abuse disorders later in life (Clark et al., 1998; Duncan et al., 1997; Grant and Dawson, 1997).

Numerous studies show that the developing adolescent brain may be more vulnerable to the effects of alcohol due to the relatively plastic nature of the adolescent CNS (Spear and Varlinskaya, 2005; Slawecki and Roth, 2004; Slawecki et al., 2004; Yttri et al., 2004). The adolescent developing CNS is characterized by significant neuronal changes in virtually every neurotransmitter system (for review see Witt, 1994; Spear, 2000). Clinical findings regarding adolescent alcohol abuse relate the above neurodevelopmental changes and adolescent alcohol exposure during this period to severe long-term functional deficits (Brown and Tapert, 2004; Brown et al., 2000). Various animal studies suggest that alcohol exposure during this sensitive period may disrupt normal neurodevelopmental processes, which may underlie changes in subsequent adult responses to ethanol (McBride et al., 2005; Sircar and Sircar, 2005; White and Swartzwelder, 2004; White et al., 2002; Crews et al., 2000).

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Previous findings from our laboratory demonstrate, that periadolescent ethanol exposure will alter the aversive properties of ethanol during a subsequent adult exposure, as measured in an ethanol-induced conditioned taste aversion (CTA; Graham and Diaz-Granados, 2001, abstract). In addition, periadolescent ethanol treatment followed by multiple withdrawal episodes (as compared to ethanol treatment followed by a single withdrawal episode) produces a greater degree of attenuation during subsequent adult ethanol-induced CTA learning (Graham and Diaz-Granados, 2001, abstract). These findings suggest that the distinct physiological state of periadolescent ethanol withdrawal may alter adult ethanol responsiveness. Diazepam (DZP), a benzodiazepine, has been shown to reduce ethanol withdrawal symptoms in rodents that experienced after ethanol exposure (Riihioja et al., 1997). In the present study, we investigated the long-term effects of periadolescent ethanol withdrawal, by administering DZP during peak withdrawal following periadolescent ethanol exposure, on a subsequent EtOH-induced CTA. We tested the long-term effects of EtOH withdrawal with the administration of DZP following periadolescent ethanol exposure on the aversive properties of ethanol during adulthood. In addition, as a method of comparison, we also investigated the effects of adult withdrawal on the aversive properties of ethanol during a subsequent (6-week delay) adult exposure.

2. General methods

2.1. Subjects

Subjects ($N=306$) were male C3H mice obtained from Charles River Laboratories (Raleigh, NC). Mice were received on post-natal day (PD) 22 and housed four to a standard Plexiglas home cage in the IACUC-approved Baylor University Neuroscience Animal Facility until the beginning of experimentation. Unless otherwise indicated, animals were maintained under a 12-h dark/light cycle with access to rodent lab chow and water ad libitum.

2.2. Route of ethanol administration

Ethanol preexposure was administered via inhalation using Plexiglas inhalation chambers ($61\times38\times61$ cm^3) (modified after Goldstein, 1972). The inhalation apparatus is designed to deliver ethanol to a volatilizing flask at a rate of 200 μl/min and then to the ethanol chamber at a rate of 7 l/min resulting in an approximate chamber ethanol concentration of 13 mg/l. Prior to the start of the experimental procedure, all treatment animals received a 1.6 g/kg ethanol loading dose which includes a 1 mmol/kg dose of pyrazole, an alcohol dehydrogenase inhibitor. Pyrazole is used to maintain and stabilize blood ethanol concentration levels (BECs). In addition, all control animals also received an initial injection of pyrazole to control for any pyrazole-related effects (Crabbe et al., 1981). Animal body weight and water intake were measured and recorded each evening prior to the administration of pyrazole and/or ethanol in order to monitor the overall health of the animals. In addition, animal body weight and water intake were also measured and recorded at the end of the exposure period.

2.3. Diazepam administration

All animals received DZP (Sigma Aldrich) treatment approximately 4 h after removal from the ethanol chamber via intraperitoneal (i.p.) injections in one of the following doses 0, 1, 2, or 3 mg/kg. Animals received DZP injections 4 h after removal from the chamber because this is the time point at which behavioral signs of ethanol withdrawal become apparent (Becker et al., 1997).

2.4. Conditioned taste aversion

Approximately 6 weeks following chronic ethanol exposure, animals were individually housed with food and water ad libitum, and allowed to acclimate to the testing environment 24 h prior to the start of the conditioning procedure. The taste aversion conditioning procedure lasted for a total of 10 days and consisted of 5 days of water restriction, 1 day of conditioning, 1 day of recovery, and 3 test days. Animal body weight was recorded daily throughout the ethanol-induced CTA procedure to monitor the overall health of the animals. Water access throughout the CTA procedure was administered in 15-ml graduated centrifuge tubes. Supplied fluids consisted of deionized water and a .15% (w/v) saccharin water solution (saccharin dissolved in deionized water).

2.5. Sampling and determination of ethanol concentrations

Blood and chamber air concentration samples were collected daily throughout the ethanol exposure period. Immediately following ethanol pre-exposure, blood samples were taken from the retro-orbital sinus for subsequent blood ethanol analysis. Blood samples were collected on ice and then diluted 50 : 1 with perchloric acid (3.4% v/v). The samples were vortexed and centrifuged at $8000\times g$. The resulting supernatant was then used in a modified enzymatic assay based on the Calbiochem-Behring method (La Jolla, CA) to determine BECs.

2.6. Statistical analysis

Saccharin consumption on the day of conditioning was compared by 2-factor ANOVA (EtOH × DZP). Within subjects saccharin consumption (conditioning day vs. test day 1) was compared for an entire group by paired t-test. Saccharin consumption during the subsequent test days was compared by 2-factor ANOVA (EtOH × DZP) with repeated measures on test session. Additional post hoc comparisons were conducted where appropriate using Fisher's Least Protected Significant Difference test (FLSD).

3. Experiment 1: periadolescent ethanol withdrawal

The purpose of Experiment 1 was to examine the long-term effects of periadolescent ethanol withdrawal (following continuous or intermittent ethanol exposure) on the associative properties of ethanol in adulthood. Periadolescent C3H mice were pre-exposed to ethanol during adolescence, 6 weeks prior to conditioning. Following ethanol preexposure, animals received DZP, a benzodiazepine, which has previously been shown to reduce the symptoms associated with ethanol withdrawal.

3.1. Specific procedures

On PD 28, periadolescent mice were randomly assigned to 1 of 16 groups receiving continuous ethanol exposure, intermittent ethanol exposure, or the appropriate control procedure. The continuous exposure treatment groups consisted of animals receiving 64 h of chronic ethanol exposure via inhalation. Approximately 4 h after removal from the inhalation chamber, animals received an i.p. injection of 0, 1, 2, or 3 mg/kg DZP. These groups are denoted as ETOHCE-D0, ETOHCE-D1, ETOHCE-D2, and ETOHCE-D3, respectively. The appropriate control groups are denoted as CONTROL-D0, CONTROL-D1, CONTROL-D2, and CONTROL-D3, respectively. These animals were treated identically to the ethanol-exposed animals with the exception of the ethanol exposure.

The intermittently exposed treatment group received 4 sessions of 16 h of chronic ethanol exposure resulting in a total of 64 h of periadolescent ethanol exposure. Thus, animals experienced 4 episodes of ethanol withdrawal (MW – multiple withdrawal), each 8 h in duration. During each of the four withdrawal periods, beginning 4 h after removal from the chamber, animals received a diazepam injection of 0, 1, 2, or 3 mg/kg DZP. These groups are denoted as ETOHMW-D0, ETOHMW-D1, ETOHMW-D2, and ETOHMW-D3, respectively. Four appropriate control groups (CONTROL-D0, CONTROL-D1, CONTROL-D2, and CONTROL-D3) experienced the exact same procedure as the ETOHMW animals with the exception of the ethanol exposure.

Following periadolescent ethanol preexposure, all animals were group housed in normal colony conditions for approximately 42 days until testing began. On approximately PD 70, animals were singly housed and allowed to acclimate for 24 h prior to the start of a 5-day water restriction schedule. During water restriction (days 1–5), animals received 30 min of unlimited access to water once daily. On the day of conditioning (CD; day 6), animals were given 15 min of unlimited access to a .15% w/v saccharin solution. Immediately following the removal of the saccharin bottles all animals received a 2.5 g/kg i.p. injection of ethanol. About 48 h following conditioning (day 8), development of a CTA was assessed by allowing animals 15 min of unlimited access to saccharin solution. Extinction, the dissociation between the CS and the US, was measured by the same test procedure for an additional 2 days in order to rule out a generalized decrement in saccharin responding (days 9 and 10; Barker and Johns, 1978).

3.2. Results

3.2.1. Blood ethanol concentrations and body weights

Weight loss and BECs corresponding to the periadolescent ethanol exposure are presented in Table 1. Blood ethanol concentrations at the time of removal from the inhalation chambers did not significantly differ among the adolescent treatment groups. Initial and final body weights did not significantly differ among ethanol exposed and control groups for all experiments. Typically, mild weight loss (3–6%) is similar for all groups undergoing the preexposure treatment.

3.2.2. EtOH-induced CTA

The complete results of Experiment 1 are presented in Figs. 1 and 2. A 2×4 ANOVA was used to analyze saccharin consumption for all treatment groups on the day of conditioning. For the ETOHCE treatment groups and respective controls, the ANOVA revealed that there were no significant differences in saccharin consumption during conditioning (see CD values in Fig. 1). An additional 2-way ANOVA revealed no significant differences in the amount of saccharin consumed on the day of conditioning for the multiple withdrawal animals (see CD values in Fig. 2). These results suggest that any differences observed in saccharin consumption throughout the remaining test days reflect a difference in experimental manipulation. To demonstrate that a significant ethanol-induced CTA occurred in all continuously exposed animals and controls, we compared by paired t-test overall saccharin consumption on the day of conditioning to overall saccharin consumption on TD1 for the entire group. Overall, animals drank significantly less saccharin on TD1, demonstrating a significant ethanol-induced CTA ($T_{124}=5.497$, $p<.001$). Additionally, all animals in the multiple withdrawal study drank significantly less saccharin on TD1, as compared to conditioning day, demonstrating a significant ethanol-induced CTA ($T_{125}=2.318$, $p=.022$).

Table 1
Blood ethanol concentration (BEC) and body weight for Experiments 1 and 2

Treatment group	BEC (mg/dl)	Initial body wt (g)	Final body wt (g)
Adolescent CECON	–	19.41±.76	19.05±.88
Adolescent MWCON	–	18.55±.54	18.25±.32
Adolescent CE64	243±.09	17.25±.40	16.28±.66
Adolescent MW64	255±.13	19.76±.18	18.6±.22
Adult CECON	–	24.42±.45	23.94±.39
Adult MWCON	–	25.13±.59	24.69±.43
Adult CE64	263±.02	26.76±.63	25.29±.8
Adult MW64	267±.014	25.45±.36	23.99±.41

Blood samples for BEC determination were taken at the time of removal from the inhalation chamber following chronic administration of ethanol. Initial and final body weights were recorded at the beginning and end of the 64-h continuous or intermittent ethanol exposure. Body weight and BEC levels corresponding to each ethanol pre-exposure are pooled across DZP treatment for clarity (no differences between DZP treatment within a group).

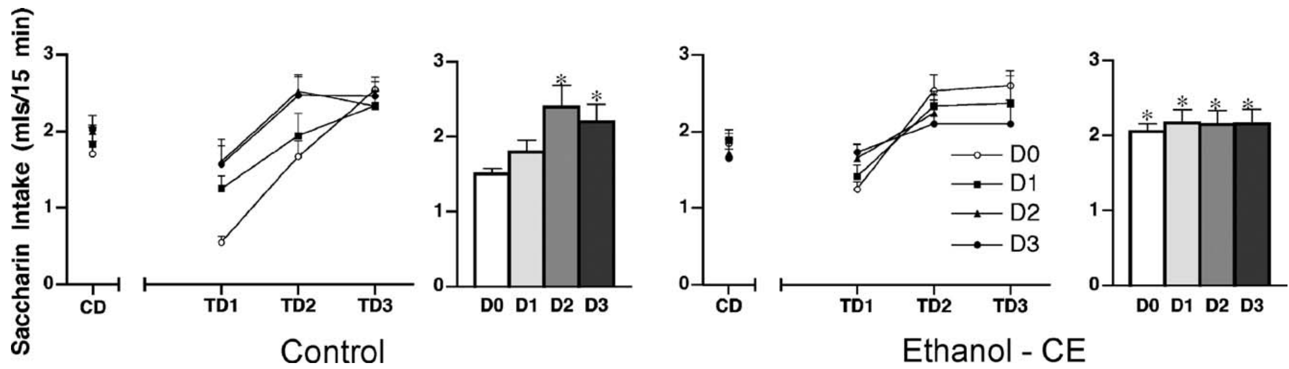

Fig. 1. The effects of continuous ethanol exposure during adolescence on the aversive properties of ethanol during a subsequent adult exposure ($N=10$–20 mice/group). Bar graphs depict between group saccharin consumption with data averaged across test days. There was no significant difference in the amount of saccharin consumed on the day of conditioning (CD). Animals pre-exposed to continuous ethanol vapor during adolescence as well as those animals that received DZP treatment only during adolescence drank significantly more saccharin than did control animals. ANOVA was used to analyze all experimental groups however, for clarity the data are depicted in separate graphs. Extinction training, the dissociation between the CS and the US, was analyzed for an additional 2 days. Asterisks indicate that saccharin consumption differs from CONTROL-D0 group (data collapsed across test days; $p<.05$).

A 2×4×3 mixed design ANOVA (EtOH×DZP×test day) was used to analyze saccharin consumption for ETOHCE animals and their controls throughout the extinction phase. The ANOVA revealed a significant overall DZP group difference, $F(3,116)=4.484$, $p<.01$, as well as a significant EtOH×DZP interaction, $F(3,116)=3.451$, $p<.05$. There was also a significant within subjects effect of test day, $F(2,232)=92.081$, $p<.001$, test day×EtOH interaction, $F(2,232)=3.074$, $p<.05$, and test day×DZP interaction, $F(2,232)=2.166$, $p<.05$. As depicted in Fig. 1, post hoc analysis using FLSD revealed a significant group difference in saccharin consumption (with data collapsed across test day) such that animals treated with EtOH drank significantly more saccharin than did control animals ($p<.01$). Thus, adult animals exposed to EtOH during periadolescence (ETOHCE-D0) demonstrated an attenuated (reduced) EtOH-induced condition taste aversion (CTA), as compared to control animals. Additionally, animals in the ETOHCE-D1, ETOHCE-D2, and ETOHCE-D3 groups also demonstrated an attenuated CTA, drinking significantly more saccharin across test days, as compared to control animals ($p<.01$). Furthermore, control animals that received diazepam treatment only (2.0 mg/kg and 3.0 mg/kg) differed significantly from the CONTROL-D0 group ($p<.01$), while animals treated with 1.0 mg/kg DZP drank comparable amounts of saccharin across the testing procedure. These data indicate that periadolescent DZP treatment alone (without EtOH) can significantly alter adult EtOH-induced conditioned taste aversion learning.

An additional 2×4×3 mixed design ANOVA (EtOH×DZP×test day) was used to analyze saccharin consumption for ETOHMW animals and their controls throughout the extinction phase (Fig. 2). The ANOVA revealed a significant overall EtOH group difference, $F(1,118)=4.639$, $p<.05$, as well as a DZP group difference, $F(3,118)=2.737$, $p<.05$. There was also a significant within subjects effect of test day, $F(2,236)=52.890$, $p<.001$, and test day×DZP interaction, $F(6,236)=2.745$, $p<.05$. While not significant, there was a strong trend toward a significant EtOH×DZP×test day interaction, $F(6,236)=2.006$, $p=.06$. As depicted in Fig. 2, post hoc analysis using FLSD test revealed an EtOH group difference in saccharin consumption such that adolescent MW animals drank

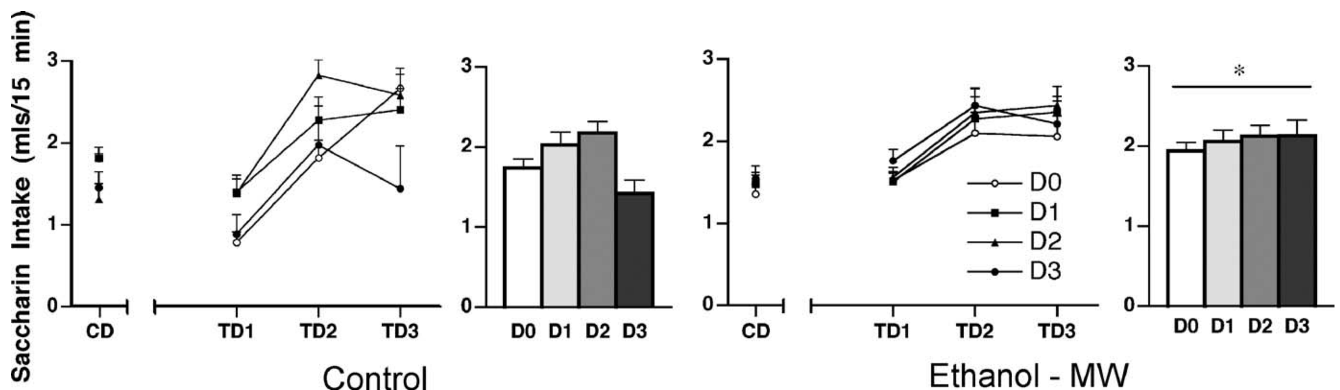

Fig. 2. The effects of multiple withdrawal episodes during adolescence on the aversive properties of ethanol during adulthood ($N=10$–17 mice/group). Bar graphs depict between group saccharin consumption with data averaged across test days. There was no significant difference in the amount of saccharin consumed on the day of conditioning (CD). Animals pre-exposed to multiple withdrawal episodes (regardless of DZP treatment) during adolescence drank significantly more saccharin than did control animals (with or without DZP). Asterisk above solid line indicates saccharin consumption in EtOH-treated animals (regardless of DZP treatment) differs from controls not treated with EtOH (regardless of DZP treatment) by FLSD ($p<.05$). ANOVA was used to analyze all experimental groups, however, for clarity the data are depicted in separate graphs. Extinction training, the dissociation between the CS and the US, was analyzed for an additional 2 days.

significantly more saccharin than did controls (data collapsed across DZP treatment and test day; $p<.05$). Additionally, adult animals treated with DZP (1.0, 2.0, and 3.0 mg/kg; data collapsed across EtOH treatment) during adolescence drank significantly more saccharin than did control animals (0.0 mg/kg) on TD1 ($p<.05$). Similar to the CE study, these findings indicate that adolescent EtOH exposure alone, as well as adolescent DZP exposure alone may reduce the aversive properties of ethanol during a subsequent adult exposure.

Previous findings from our laboratory indicate that adolescent ethanol treatment followed by multiple withdrawal episodes (as compared to ethanol treatment followed by a single withdrawal episode) produces a greater degree of attenuation during subsequent adult ethanol-induced CTA. In the present study, the two adolescent EtOH treatment groups (CE and MW) drank significantly different amounts of saccharin during conditioning, making any subsequent saccharin intake comparisons impossible. However, results indicate that adult animals exposed to continuous ethanol vapor (one withdrawal episode) during adolescence drank approximately 30% less saccharin on TD1 (as compared to CD), while adult animals exposed to multiple withdrawal episodes during adolescence drank approximately 10% more saccharin on TD1 (as compared to CD). On average, control animals (CECON and MWCON) drank approximately 60–70% less saccharin on TD1. These data are consistent with our previous findings and suggest that adolescent MW treatment produces less of an aversion than adolescent CE treatment.

4. Experiment 2: adult ethanol withdrawal

4.1. Specific procedures

This experiment was identical to the first experiment with the exception that ethanol preexposure followed by treatment with DZP was administered during early adulthood. On PD 70, adult mice were randomly assigned to 1 of 16 treatment groups. Treatment groups included ETOHCE-D0, ETOHCE-D1, ETOHCE-D2, ETOHCE-D3, CONTROL-D0, CONTROL-D1, CONTROL-D2, CONTROL-D3, ETOHMW-D0, ETOHMW-D1, ETOHMW-D2, ETOHMW-D3, CONTROL-D0, CONTROL-D1, CONTROL-D2, and CONTROL-D3. About 6 weeks after ethanol exposure the associative properties of ethanol were assessed. The conditioning procedure was identical to the CTA procedure described in Experiment 1, which consisted of 5 days of water restriction, 1 day of conditioning, 1 day of recovery, and 3 test days.

4.2. Results

4.2.1. Blood ethanol concentrations and body weights

Weight loss and BECs corresponding to the adult ethanol exposure are presented in Table 1. Blood ethanol concentrations at the time of removal from the inhalation chambers did not significantly differ among the adult treatment groups. Initial and final body weights did not significantly differ among ethanol exposed and control groups for all experiments. Typically, mild weight loss (3–6%) is similar for all groups undergoing the ethanol pretreatment.

4.2.2. EtOH-induced CTA

The complete results of Experiment 2 are presented in Figs. 3 and 4. A 2×4 ANOVA was used to analyze the amount of saccharin consumed during conditioning for the continuously exposed animals and multiple withdrawal animals. No significant differences were found during conditioning for all groups. Therefore, any differences observed in saccharin consumption throughout the remaining test days reflect a difference in experimental manipulation. In addition, paired t-test analysis across the entire group was conducted to analyze saccharin consumption between conditioning day and test day 1. Animals displayed a significant reduction in saccharin consumption on TD1 as compared to CD ($T_{77}=6.987$, $p<.001$ and $T_{81}=5.944$, $p<.001$, for CE and MW animals, respectively). These data indicate that adult animals treated with ethanol 6 weeks prior to conditioning, as well as control animals not exposed to ethanol display a significant CTA.

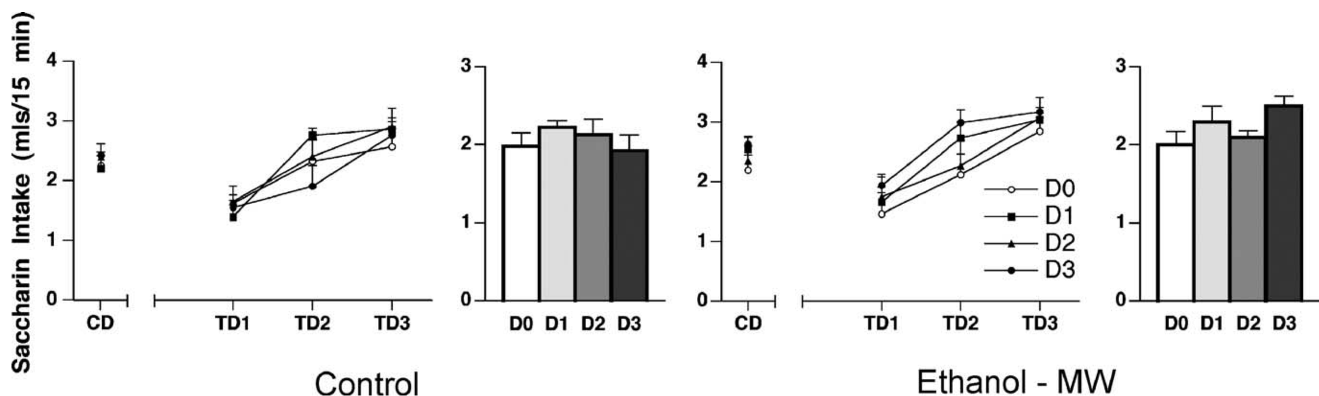

Fig. 3. Continuous ethanol exposure in adult animals has no effect on the aversive properties of alcohol during a subsequent EtOH-induced taste aversion ($N=8$–10 mice/group). Bar graphs depict between group saccharin consumption with data averaged across test days. All animals drank similar amounts of saccharin on the day of conditioning (CD). All animals displayed a similar degree of ethanol-induced conditioned taste aversion as evidenced by a significant decrease in consumption on TD1 as compared to conditioning day.

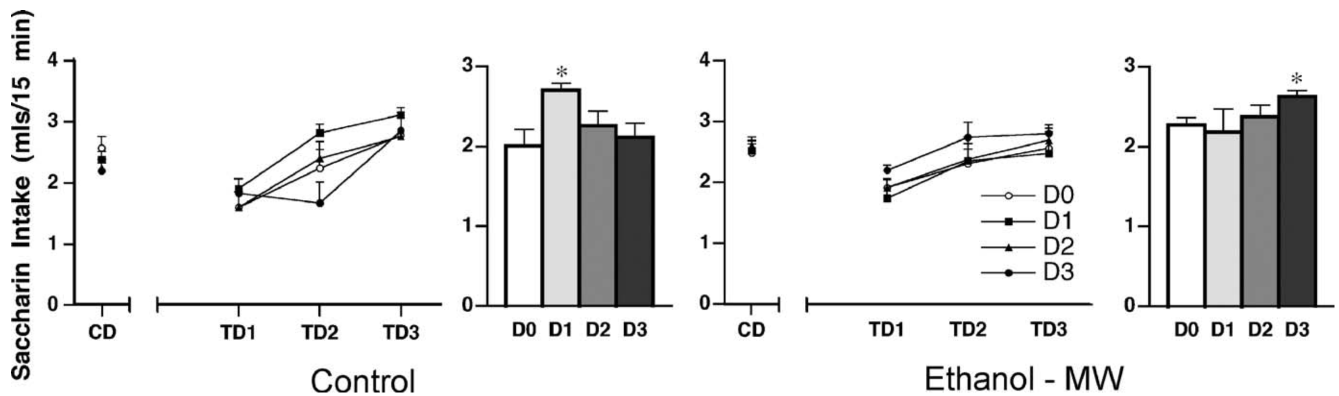

Fig. 4. The effects of multiple withdrawal episodes during adulthood on the aversive properties of ethanol during a subsequent adult EtOH-induced taste aversion ($N=8$–10 mice/group). Bar graphs depict between group saccharin consumption with data averaged across test days. All animals drank similar amounts of saccharin on the day of conditioning (CD). Asterisk indicates ETOHMW-D3 and CONTROL-D1 differ in saccharin consumption from MWCON-D3 ($p<.05$).

Two separate 2×4×3 mixed design ANOVAs (EtOH× DZP×test day) were used to analyze saccharin consumption throughout the extinction phase for both EtOH exposure paradigms (CE and MW). In the case of adult animals exposed to continuous ethanol vapor, the ANOVA revealed no significant EtOH group effect, no significant DZP group effect, or any significant EtOH×DZP interaction (Fig. 3). There was, however, a significant within subjects effect of test day, $F(2,136)=29.725$, $p<.001$, indicating that animals displayed extinction, the disassociation between the CS and US.

In the case of adult animals exposed to multiple withdrawal episodes, followed by DZP treatment, the ANOVA (EtOH× DZP×test day) revealed no significant between group effect, but a significant EtOH×DZP interaction, $F(3,74)=3.154$, $p=.030$. As depicted in Fig. 4, subsequent analysis of main effects (data collapsed across test days) revealed that animals treated with CONTROL-D3 drank significantly less saccharin than did adult animals in the ETOHMW-D3 group and CONTROL-D1 group ($p<.05$). This result is rather difficult to explain, but may be due to the fact that animals in that MWCON-D3 group drank slightly less saccharin on TD2 as compared to TD1, an unusual result during extinction learning in a one-trial taste aversion procedure. As was expected, there was also a significant within group effect of time, $F(2,148)=34.196$, $p<.001$, indicating that animals displayed extinction learning.

5. Discussion

Adult C3H mice experiencing withdrawal (single or multiple episodes), DZP treatment only, or EtOH exposure followed by DZP treatment during the period of periadolescence demonstrated a reduced ethanol-induced CTA (less of an aversion), as compared to animals that received no EtOH exposure during periadolescence. Alternatively, adult animals experiencing withdrawal (single or multiple episodes), chronic EtOH followed by DZP, or DZP alone displayed an EtOH-induced CTA that was indistinguishable from untreated controls. Based on these findings, it is apparent that adolescent, but not adult, ethanol and/or DZP exposure experienced 6 weeks prior to a subsequent exposure can produce long-lasting changes in the aversive properties of ethanol. This is consistent with a previously published report indicating that periadolescent EtOH exposure increases the reinforcing properties of ethanol (Rodd-Henricks et al., 2002).

Given that multiple withdrawal episodes further attenuates the conditioning of aversive properties associated with EtOH (Graham and Diaz-Granados, 2001, abstract), the primary objective of the present experiment was to investigate the role of withdrawal experience on the long-term effects of periadolescent ethanol exposure. Our approach was to administer DZP, a drug previously shown to reduce withdrawal symptoms (Riihioja et al., 1997), during peak ethanol withdrawal. Surprisingly, periadolescent DZP treatment, alone or in combination with EtOH, was sufficient to reduce the aversive properties of ethanol during a subsequent adult exposure. Therefore, rather than ameliorating withdrawal and lessening the attenuation of the conditioned taste aversion, DZP administration during the periadolescent period mimicked the long-term effect of an attenuated EtOH-induced CTA. It is important to note that in contrast, the administration of EtOH/DZP or DZP only during adulthood, experienced 6 weeks prior to conditioning, had minimal effect on adult CTA, suggesting that the adolescent developing CNS is more susceptible to the long-lasting effects of EtOH and/or DZP exposure. Given the common mechanism of action between EtOH and DZP, it is reasonable to surmise that EtOH-induced changes in the periadolescent GABA system are involved in the long-term effects of periadolescent EtOH exposure on the aversive properties of EtOH in adulthood.

To date, benzodiazepine administration remains one of the most effective treatments in the management of alcohol withdrawal (Manikant et al., 1993; Mayo-Smith, 1997; Addolorato et al., 1999). Chronic benzodiazepine treatment will produce withdrawal symptoms similar to that observed during ethanol withdrawal (Hallstrom and Lader, 1981; Rickels et al., 1983; Greenblatt et al., 1983; Korpi et al., 1997) and benzodiazepine withdrawal can occur following high doses as well as moderate and low doses of benzodiazepines (Hallstrom and Lader, 1981). The ability of benzodiazepines to suppress

alcohol withdrawal symptoms may be due to their anticonvulsant and anxiolytic properties or their ability to fully substitute for EtOH. Many human alcoholics indicate that alcohol and benzodiazepines (specifically DZP) are used interchangeably and produce similar effects (Kostowski and Bienkowski, 1999). Similar discriminative or subjective effects of certain recreational drugs contribute to the initiation of drug use and/or relapse (Stolerman, 1992). In animals, the discriminative properties of certain benzodiazepines exhibit complete substitution for ethanol (Kostowski and Bienkowski, 1999; Bienkowski et al., 1997; Lytle et al., 1994). One possible interpretation of the findings presented here is that periadolescent EtOH followed by DZP, or DZP alone, produced subjective effects similar to that experienced during a subsequent adult EtOH exposure, thereby producing an attenuated adult ethanol-induced CTA. Therefore, it is reasonable to suggest that periadolescent animals exposed to DZP (either alone or following EtOH) experienced benzodiazepine withdrawal, a phenomenon known to be physiologically and behaviorally similar to that of alcohol withdrawal. Additional studies are needed to further investigate this possibility.

Another interpretation of our findings may be that tolerance and/or cross-tolerance exists between ethanol and DZP following periadolescent EtOH/DZP exposure. Neonatal, perinatal, or periadolescent EtOH exposure has been shown to produce tolerance during subsequent EtOH testing (Little et al., 1996; White et al., 2000; Silveri and Spear, 2001). For example, adult animals chronically exposed to EtOH during the periadolescent developmental period display greater tolerance to the ataxic effects of a subsequent EtOH challenge dose (Diaz-Granados et al., 1999, abstract). Similarly, adult animals chronically exposed to benzodiazepines also display tolerance to the sedative effects of the drug during a subsequent BZD challenge (Fernandes et al., 1999; File, 1986). Although there are no known investigations of the effects of periadolescent BZD exposure on adult tolerance, a number of findings also indicate that neonatal or perinatal BZD exposure will produce benzodiazepine tolerance in mature animals (File, 1986). Furthermore, chronic EtOH exposure will produce cross-tolerance to certain benzodiazepines and barbiturates (Toki et al., 1996; Newman et al., 1986; Curran et al., 1998), and benzodiazepine dependent animals display cross-dependence to ethanol (Chan et al., 1988, 1990; Khanna et al., 1998). Thus, it is possible that one of the effects of either EtOH or DZP administration during the periadolescent period is a lasting tolerance and/or cross-tolerance decreasing the aversive properties of either agent during a subsequent adult exposure. Further investigations into the effects of EtOH and/or DZP exposure during periadolescence on the development of tolerance to both agents as well as other similar agents are warranted.

Specific behavioral and pharmacological studies show that some of the deleterious effects of ethanol are more pronounced in periadolescent animals as compared to adult animals (York and Chan, 1994; Silveri and Spear, 2000, 2001). For example, periadolescent animals as well as periadolescent humans are more sensitive to the memory-impairing effects of alcohol than are adults (Markwiese et al., 1998; White et al., 2000; Acheson et al., 1998). Thus, a possible explanation for the present findings may be that periadolescent EtOH and/or DZP administration induced long-term learning impairments. This possible explanation is supported by studies of long-term potentiation and NMDA-mediated activity in hippocampal slices showing that periadolescent neurons are more sensitive to ethanol inhibition than adult neurons (Swartzwelder et al., 1995a,b). However, in contrast, other behavioral studies indicate that periadolescent animals are less sensitive to the deleterious effects of ethanol. For example, periadolescent animals displayed less motor impairment and sedation following high ethanol challenge doses which typically produce loss of the righting reflex in adult animals (Moy et al., 1998; Silveri and Spear, 1998; Little et al., 1996). In fact, recent studies investigating the long-term effects of periadolescent ethanol exposure on associative learning indicate that periadolescent ethanol does not produce deficits in associative learning mechanisms responsible for CTA learning (Yttri et al., 2004). Therefore, it is unlikely that the attenuated CTA observed in this study is a result of periadolescent ethanol-induced learning impairments.

Clearly, exposure to EtOH, EtOH followed by DZP, or DZP alone during the periadolescent developmental period reduced the aversive properties of ethanol during a subsequent adult exposure. However, exposure to the same drugs during adulthood produced minimal effects. Therefore, the most plausible explanation for the present finding is that exposure to EtOH and DZP during the periadolescent developmental period results in a long-term change in the normal development of the CNS. There is substantial evidence confirming that the periadolescent brain is distinct from that of the adult brain (Spear, 2000). A multitude of molecular and physiological changes occur during the period of adolescence. Specific changes include a substantial reduction in the number of synaptic connections, alterations in certain receptor levels, most notably GABA-A, and fluctuations in neurotransmitter levels (Rakic et al., 1994; Laurie et al., 1992). GABA dependent benzodiazepine receptors in the cerebral cortex are present on PD 1, undergo substantial increases during the first week of life and then decrease to adult levels during the 4th week of postnatal development (Lippa et al., 1981). Furthermore, the alpha subunit of the GABA-A receptor, which is thought to mediate benzodiazepine binding, has also been shown to exhibit postnatal changes during the first month of life (MacLennan et al., 1991). Again, it is plausible that normal developmental processes are disrupted by EtOH and/or DZP during adolescence resulting in long-lasting modifications with respect to subsequent EtOH responsiveness.

In addition to the normal developmental changes seen during the periadolescent developmental period, ethanol interacts with the developing adolescent CNS in a manner distinct from that of the mature brain. For example, binge drinking-induced brain damage in specific brain regions is greater in adolescent animals as compared to adult animals (Crews et al., 2000), and repeated ethanol exposure produces significant age-dependent changes in basal mesolimbic dopamine levels (Philpot and Kirstein,

2004). While there are no known published studies investigating the long-term neurobehavioral effects of DZP or EtOH/DZP administration during the period of adolescence, neonatal and perinatal DZP administration is known to produce long-term behavioral and physiological effects (Schroeder et al., 1994; Miranda et al., 1990; File, 1986). Due to the relatively plastic nature of the periadolescent CNS, it is possible that DZP exposure (or the combined exposure of EtOH and DZP) during adolescence produces similar alterations in BZD receptor sensitivity, resulting in a reduced aversion to EtOH during adulthood. The above ontogenetic findings, taken together with additional research demonstrating that EtOH and DZP interact with the GABA-A receptor complex, and that the interaction of EtOH and DZP with the GABA-A receptor complex varies during development, suggest that periadolescent exposure to these drugs may produce long-term changes at the level of the GABA-A receptor, thereby altering EtOH responsiveness during adulthood.

These findings demonstrate that EtOH exposure as well as EtOH exposure followed by DZP treatment during adolescence, but not adulthood, can alter EtOH responsiveness during a subsequent adult exposure. In addition, the administration of DZP alone during adolescence is sufficient to produce long-term changes in ethanol responsiveness during adulthood. Currently, alcohol is still the drug of choice among junior high and high school students. Young individuals are also experimenting with other depressive agents, including Valium (Diazepam; National Household Survey on Drug Abuse, 2002). Although the objective of the present study was not to investigate the long-term effects of adolescent diazepam exposure, it is important to note the findings presented here suggest that the recreational use of alcohol and/or Valium (together or alone) during the periadolescent period of neurodevelopment may lead to long-term neurological changes. These long-term neurological changes can subsequently produce impairments in the adult response to alcohol and other drugs of abuse.

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